IN VIVO AND IN VITRO METHODS OF SELECTING FOR TOLERANCE TO THE HERBICIDE 2,4-D IN RED CLOVER (*Trifolium pratense* L.).

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1987

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to the chairman of the supervisory committee, Dr. Kenneth H. Quesenberry, for hours of valuable guidance and assistance. In addition, Dr. Quesenberry is thanked for providing technical and financial support for the research project.

Dr. David D. Baltensperger is greatly appreciated for his friendship and his involvement in my education, as well as for his countless contributions as a committee member. I would also like to thank Dr. Joan Dusky, Dr. Jude Grosser and Dr. David Hall for participating on the supervisory committee and contributing their valuable time and comments. I am also grateful to Dr. Donn Shilling for his enthusiasm, interest and ideas.

The work mentioned in this dissertation could not have been accomplished without the expert technical help of Mr. David Moon and many other research assistants and graduate students, each of whom are thanked.

I am greatly indebted to my parents, Dr. and Mrs. Norman L. Taylor, and other family members, for providing the necessary love, advice and support that made my graduate education possible.

My deepest appreciation goes to my wife, Belinda, whose love, help, patience, understanding, sacrifices, and endurance often went unrewarded. It was through Belinda's kindness, reassurance and support that I was able to undertake and complete a graduate education. Finally, I am most thankful for my son, Ryan, whose smile makes it all worthwhile.

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By

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August, 1987

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Major Department: Agronomy

The objective of this research was to develop a 2,4-D [(2,4-dichlorophenoxy) acetic acid] tolerant red clover (Trifolium pratense L.) population, using two contrasting techniques. Recurrent half-sib family selection was used to develop increasingly more tolerant plant populations. At the cellular level, variant cell types were selected for growth in the presence of high levels of 2,4-D with the intention of regenerating 2,4-D tolerant individual plants.

Four cycles of recurrent selection increased levels of 2,4-D tolerance in red clover by approximately 35%. Family selections were based on means obtained through the use of a 1-5 visual rating scale. The narrow-sense heritability of the 2,4-D tolerance trait was high, averaging nearly 50%

and inbreeding depression was kept to a minimum. These variables indicated that significant progress had been made and that future progress should be possible. Several more cycles of selection must be conducted to obtain 2,4-D tolerance levels high enough for use in a pasture situation. The agronomic potential of 2,4-D populations must also be investigated.

In vivo and in vitro responses to 2,4-D of eight red clover genotypes were shown to be correlated ($r = 0.77$). This provided justification for using in vitro systems to select for 2,4-D tolerance. Suspension cultures of a regenerative genotype were plated onto agar-based nutrient media supplemented with 40 mg L^{-1} 2,4-D in the selection experiments. Sixteen 2,4-D tolerant cell lines were identified after two cycles of selection. These lines were evaluated for 2,4-D tolerance using a 2,4-D bioassay procedure. Callus tissues, after having been on nutrient media supplemented with 40 mg L^{-1} 2,4-D, were placed on the top of oat (*Avena sativa* L.) coleoptile or internode sections. Susceptibility was indicated by the amount of oat section elongation after 24 hours. Two of the more tolerant lines proved to have 86% and 97% less 2,4-D in their tissues than the susceptible control tissue. The tolerant lines have not responded to regeneration attempts.

CHAPTER I

INTRODUCTION

Crop production systems rely heavily upon various herbicides for weed control. Innovations in equipment and technology bring about changes in production practices that may require unique methods of weed control. The environmental fate and toxicity of herbicides is continually being studied, resulting in the removal of certain products from the market. Weeds are developing genetic tolerances via natural selection in crop monocultures where specific herbicides are repeatedly applied. For these reasons, producers of today's agricultural products require the rapid development of safer, more effective, and more selective herbicides.

It is becoming more difficult for chemical companies to develop new products to meet these needs. New formulations may take many years and millions of dollars to research, evaluate and market. Environmental constraints and safety restrictions have made the registration of new products more difficult. As a result, there is a current trend for fewer new herbicides to be developed each year (Faulkner,

1982). Chemical companies need and are searching for alternatives. One such measure would be to make better use of existing herbicides.

If agronomically-useful crop cultivars could be developed that express tolerance to certain herbicides, then this could potentially increase the use of currently marketed herbicides, thereby decreasing the demand for new herbicides. Economically, it could be more feasible to develop crops tolerant to specific herbicides than to formulate new products. Faulkner (1982) estimated the cost of developing tolerant cultivars to be in the range of one to five percent of the expenses involved with new herbicides.

Cultivars tolerant to broad-spectrum herbicides, such as 2,4-D [(2,4-dichlorophenoxy) acetic acid], would be especially useful as they could be employed in a variety of environments. The intention of this research was to incorporate 2,4-D tolerance into red clover (Trifolium pratense L.). Red clover is an important forage legume throughout many parts of the world. Red clover is believed to have originated in southeastern Europe and Asia Minor and it is now grown in many temperate regions of the world (Taylor and Smith, 1979). In the United States, red clover is grown as a short-lived perennial in the northeastern and central geographic regions. In the southeastern United

States, red clover is generally grown as a winter annual (Smith et al., 1985). Red clover can be used for hay or grazing and is usually grown in association with a forage grass, such as timothy (Phleum pretense L.) (Taylor and Smith, 1979).

Weed control in established, mixed pastures is often a problem. Due to the relatively small market, chemical companies rarely develop selective herbicides for pasture crops. Red clover cultivars tolerant to 2,4-D would be beneficial in mixed pasture situations as the companion grass would not be damaged upon a postemergence spray application of 2,4-D and nearly all broadleaf weeds could be controlled. The development of a 2,4-D tolerant red clover cultivar appears to be an economic alternative to the development of selective herbicides.

Conventional plant breeding techniques have traditionally been applied as a means of obtaining herbicide tolerant cultivars (Devine et al., 1975). Recently, tissue culture systems have emerged as an alternative to the sometimes laborious conventional methods of breeding (Meredith and Carlson, 1982). This project examined both systems of selecting for herbicide tolerance. Recurrent half-sib family selection was used to develop increasingly more tolerant plant populations. At the

cellular level, variant cell types were selected for growth in the presence of high levels of 2,4-D with the intention of regenerating 2,4-D tolerant individual plants from tolerant callus cultures

CHAPTER II

REVIEW OF LITERATURE

Breeding for Herbicide Tolerance using Conventional Methods

Intraspecific Variation in Sensitivity to 2,4-D

Shortly after the discovery of 2,4-D [(2,4-dichlorophenoxy) acetic acid] during World War II, and subsequent delineation of its phytotoxic effects, numerous reports appeared regarding differential intraspecific tolerances. Albrecht (1947) measured the degree of 2,4-D tolerance in 66 different creeping bentgrass (Agrostis palustris Huds.) strains. The range of responses varied from slight injury to severe injury with no stolon recovery. His was the first such report of intraspecific variation in tolerance to 2,4-D. Many reports have followed describing differential 2,4-D sensitivity of an abundance of crop and weed species.

Tandon (1949) found several cultivars of flax (Linum usitatissimum L.) to be more tolerant to 2,4-D than others in regard to grain yield, maturity, 1,000 seed weight, stem curvature, and oil percentage. Fantals and Dunham (1949)

also used the stem curvature response as a measure of tolerance in flax and found significant cultivar differences. Inbred lines and hybrids of corn (Zea mays L.) have also been shown to have varying degrees of tolerance to 2,4-D. Roseman and Stanlforth (1949) found that, when treated at the 6 to 8 leaf stage, inbred lines displayed large differences in susceptibility to 2,4-D. Oliver (1949) found that parent corn lines exhibited more tolerance than their hybrids. Elder and Davies (1948) found a differential response among sorghum [Sorghum bicolor (L.) Moench.] varieties for grain and dry forage yield.

Clovers (Trifollum spp.) also differ in response to 2,4-D. Stryckers (1958) found a strain of white clover (T. repens L.) that could not be controlled in a Belgian pasture following nine 2,4-D treatments. Taylor et al (1982) evaluated 2,4-D tolerance of white clover cultivars and rated them on a 1-10 scale (10=dead). 'Regal' and 'Sacramento' were most susceptible (rating 7.8) while 'Lucky' exhibited the most tolerance (rating 4.5). They also tested red clovar (T. pratense L.) and found the breeding line K4-183 to be the most tolerant (rating 5.5) and K4-184 to be the most susceptible (rating 7.3). Henderson and Clayden (1983) screened 480 red clover cultivars for tolerance to 2,4-D. Eleven of these were selected for tolerance and placed in a breeding program

They also found that on a whole, diploid red clover lines were more tolerant than tetraploid lines.

Ssansy and Hanson (1970) made the observation that cultivars of birdsfoot trefoil (Lotus corniculatus L.) differ in response to 2,4-D. They suggested that genetic systems were responsible for the tolerance that was observed.

Luckwill and Cassley (1966) found that the ability to detoxicate 2,4-D or to oxidize the acetic acid sidechain to CO_2 varies widely between apple (Malus sylvestris Mill.) cultivars. 'Cox' is capable of degrading 2,4-D and thus has tolerance. 'Bramleys Seedling' cannot degrade 2,4-D and is susceptible. Similar results have also been demonstrated with strawberry (Fragaria spp.) (Luckwill and Lloyd-Jones, 1960).

Significant differences in sensitivity to 2,4-D have been reported for winter wheat (Triticum aestivum L.) (Swan and Rohde, 1962), spring wheat (Rohmont, 1948; Warden and Mullette, 1949), barley (Hordeum vulgare L.) (Darschaid et al., 1952), oats (Avena sativa L.) (Robinson et al., 1950), rice (Oryza sativa L.) (Sundaru et al., 1953), soybean (Glycine max (L.) Merr.] (Ennis et al., 1952), and sugarcane (Saccharum spp.) (Nolla, 1950).

Differential response to 2,4-D is also a recognized phenomenon among numerous weed species. Whitehead and Switzer (1963) found a strain of wild carrot (Daucus carota

L.) that could no longer be controlled by 2,4-D. It was discovered in Ontario, Canada along roadsides that had been repeatedly sprayed with 2,4-D. Resistant and susceptible strains were identified that were morphologically identical. The 2,4-D resistant strain represents less than one percent of an unsprayed population. Continued spraying increases the percentage of the resistant type.

Bell et al. (1973) reported differential tolerances of perennial sowthistle (Sonchus arvensis L.). The susceptible type was crossed with a tolerant annual sowthistle (S. oleraceus L.). The hybrids and backcrosses to the annual proved to have more tolerance than the original susceptible parent. This genetic basis of 2,4-D tolerance and the natural interspecific crosses between the sowthistles are suggested explanations for the detailed variability in 2,4-D tolerance.

Other weeds with documented reports of intraspecific variation in sensitivity to 2,4-D include common lambsquarters (Chenopodium album L.) (Bemuge, 1974), kochia (Kochia scoparia (L.) Schrad.) (Bell et al., 1972), spreading dayflower (Commelina diffusa Burm.) (Hilton, 1957), Russian knapweed (Centaurea repens L.) (Stallings and Cords, 1969), field bindweed (Convolvulus arvensis L.) (Whitworth and Mazik, 1967), Canadian thistle (Cirsium arvense (L.) Scop.) (Hodgson, 1964), hoary cress (Cardaria chalepensis (L.) Hand.-Maz.), (Sexsmith, 1964), yellow

nutsedge (Cyperus esculentus L.) (Costs and Appleby, 1976), and fireweed [Erechtites hieracifolia (L.) Raf.] (Hanson, 1962).

Inheritance of Tolerance to Herbicides

Scientists have taken advantage of the wealth of variation in reaction of plant species to 2,4-D and other herbicides. This variation provides excellent material for biochemical studies, the determination of modes of action of certain herbicides, and offers the opportunity to study morphological and taxonomic relationships. Some have addressed the genetic aspects and applied breeding techniques to determine how these characters are controlled. Wiese and Quimby (1969) crossed resistant and susceptible sorghum plants. In the F_3 generation they found 75 percent of the segregates to be similar to the resistant parent, indicating that resistance to 2,4-D was controlled by a single dominant gene. Mothum (1954) obtained similar results in a cross of two flax cultivars. He used the degree of stem curvature as a measure of susceptibility. He found that the stem curvature response was simply inherited and determined that there was a significant phenotypic correlation between F_2 plant ratings and the means of the derived F_3 lines.

A phenoxy herbicide related to 2,4-D, MCPA [(4-chloro-2-methylphenoxy) acetic acid], is also used for broadleaf weed control. Stafford et al. (1968) studied the inheritance of MCPA tolerance in flax and attempted to determine the feasibility of incorporating this tolerance into commercial cultivars. Crosses between tolerant and susceptible cultivars were evaluated in the F_2, F_3 and backcross generations. Relatively low heritability values were obtained due to the influence of the environment and low genetic variance. Change in anthocyanin production and stem curvature were shown to be two of the most heritable traits and the authors suggest that crop improvement could be moderately effective if these two traits were selected.

Faulkner (1974) studied the heritability of paraquat [1-1'-dimethyl-4,4'-bipyridinium ion] tolerance in perennial ryegrass (Lolium perenne L.). Faulkner detected a quantitative pattern of inheritance using visual ratings and yields as measurements of tolerance. Narrow-sense heritability estimates were relatively high, in the range of 50 to 72 percent. He found that visual ratings were nearly as effective as yield measurements and concluded that it would be possible to increase tolerance to paraquat by selection.

Metribuzin [4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4H)-one] tolerance has been studied for several crop species. De Jong (1983) found

that sensitivity was controlled by a single recessive gene in cultivated diploid potatoes (Solanum tuberosum L.). Edwards and Kilen (1976) obtained similar results in soybean with 'Semmes'. Bugg (1983) also found a recessive gene to be responsible for susceptibility to metribuzin in soybean but he also determined that more than one gene was involved in two other crosses. A major gene with modifiers was found by Souza Machado et al. (1982) to be in control of metribuzin tolerance in tomato (Lycopersicon esculentum Mill.).

Atrazine [6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine] tolerance has been extensively studied. Grogan et al. (1963) found a Mississippi selection of corn to be susceptible to the herbicide and subsequently determined that tolerance was controlled by a single major dominant gene. Scott and Grogan (1969) used F_2 populations from 16 reciprocal translocation crosses to locate the susceptible gene to the long arm of chromosome 8. In contrast, Comstock and Anderson (1968) found a quantitative pattern of atrazine tolerance in flax. Variation was attributed to environmental factors and suggested that selection would be difficult.

Breeding Herbicide-Tolerant Crop Cultivars

The ultimate goal of plant breeders working with herbicide tolerance is the development of herbicide-tolerant crop cultivars. This objective has best been exemplified by Faulkner who has been a leading proponent of the registration of agronomically-useful cultivars tolerant to specific herbicides. Faulkner (1975) developed and registered a paraquat tolerant line of *L. perenne* ('Causeway') by recurrent selection, but unfortunetely it did not yield well (Faulkner, 1976). Wright and Faulkner (1982) combined the paraquat tolerance of Causeway with a high performance cultivar called 'Aberystwyth S23'. Ten plants of Causeway were paired and mated with ten plants of Aberystwyth S23. The resulting progeny were sprayed with paraquat. Six of the strongest seedlings from each cross were intermated and F_2 seed was harvested in bulk. Intensive selection was practiced on the F_2, F_3 and F_4 generations. The F_4 population was significantly more paraquat tolerant than the tolerance exhibited by Causeway. At the time of the publication, yield trials were underway to determine the agronomic potential of the population. Faulkner (1978) also selected perennial ryegrass lines that were tolerant to the herbicide dalapon (2,2-dichloropropanoic acid). 'Rathlin' not only has dalapon tolerance but also has good agronomic characteristics. Recurrent selection was also used by

Wright and Faulkner (1982) in selecting for glyphosate [N-(phosphonomethyl) glycine] tolerance in perennial ryegrass. The original population consisted of 5,000 seedlings of six different cultivars. These were sprayed and the survivors were mated in six polycross groups representing the six cultivars. Four thousand progeny of each of these groups were sprayed for another cycle of selection. Thirty plants were selected and intermated in two polycross groups. The two populations selected were 50% and 57% more tolerant than the controls.

Phenotypic recurrent selection was used by Devine et al. (1975) to develop a line of birdsfoot trefoil tolerant to 2,4-D. Progeny testing allowed for the selection of 34 clones from an original population of 75. These 34 clones were intercrossed, progeny were established in the field and sprayed with 1.7 to 2.4 kg ha^{-1} 2,4-D. Selections were based on ratings of regrowth. Five cycles of field selection were conducted. Evaluation tests determined that selection for 2,4-D was effective as tolerance was gained. Fresh weight of one of the lowest yielding tolerant progeny lines was still five times that of a susceptible cultivar after 2,4-D application. Survival percentage, dry weight, plant nitrogen composition and surface wax accumulation were also higher in the tolerant strain. The tolerant strain exhibited the same early symptoms of damage as the susceptible strain (stunting and stem curvature) but

recovered quicker. Despite the level of tolerance, no report has been seen regarding the agronomic quality of the tolerant line nor has there been a cultivar released (Faulkner, 1982).

Henderson and Claydon (1983) selected 11 lines that were tolerant to 2,4-D from an evaluation test containing 480 red clover lines. Selections from eight of the best eleven were cross-pollinated in isolation cages. Tolerance after one cycle was evaluated and found to be increased.

Miller et al. (1984) have also bred for tolerance to 2,4-D. Three cycles of recurrent selection in arrowleaf clover (T. vesiculosum L.) resulted in a two-fold increase in 2,4-D tolerance as compared to the susceptible controls. Tolerance to 2,4-D was significantly correlated with seedling vigor and growth habit.

Summary and Conclusions

There is a shortage of cultivars that have been registered as being tolerant to certain herbicides. The amount of variation present, described in the first section of this chapter, for tolerance to 2,4-D should provide ample opportunity for selection and progress to be made. There are equal amounts of documented variation in sensitivity to other herbicides (see book by LeBaron and Gressel, 1982). Inheritance studies have been conducted on this variation and some indicate qualitative control of

tolerance. It seems only logical that the next step would be germplasm development but this step seems only to have been taken seriously by Faulkner (1975, 1978).

Recurrent selection has been used successfully in cases where attempts to develop herbicide tolerance have been made. Taylor et al. (1985) suggest recurrent selection as a tool to develop genotypes that have not been seen in previous populations. This suggests that even with a low level of initial herbicide tolerance in a particular crop species, one should be able to develop higher levels of tolerance without resorting to alternate sources of variation (i.e., somaclonal, interspecific hybridization, mutation, etc.). The problem, as stated by Taylor et al. (1985), is breeding for one trait without simultaneously introducing other undesirable characteristics into the population.

Selection for Herbicide Tolerance in Plant Cell Cultures

In Vitro Selection Techniques

New methods of breeding for herbicide tolerance are rapidly being developed and employed. These techniques involve selection for herbicide tolerance at the cellular level via tissue cultures and cell suspensions. These techniques allow for the screening of millions of

potentially different cells in a single flask. Gressel (1979) considered the advantages of screening in cell cultures over screening at the whole plant level. He suggests that cell cultures are superior because the number of escapes could be reduced, due to the greater uniformity of the culture environment. The selection pressure imposed in the field can rarely be made to exceed 90 to 95 % kill and many of the remaining plants are likely to be escapes. Increasing the herbicide rate will likely kill the truly tolerant plants in the field. Field selection is also considered to be laborious, expensive and time consuming (Crocomo and Ochoa-Alejo, 1983).

Another advantage of cell culture selection is the belief that cell culture is a novel source of genetic variation. The expression of variant phenotypes regenerated from tissue grown in cultures has been termed somaclonal variation (Larkin and Scowcroft, 1981). Plants have been cultured in the past mainly as a means of cloning a particular genotype that is vegetatively propagated. All such clones were expected to be identical. Phenotypic variants were quite generally ignored and dismissed as artifacts of tissue culture (Larkin and Scowcroft, 1981) As more and more tissue culture work has been conducted, it has become increasingly obvious that these variants are common and may be of potential value to the plant breeder, as variability is the basis of crop improvement. Somaclonal variation has been attributed to the

preexisting variation in the explant donor tissue or to induced changes in the cultured tissue. These induced changes can be caused by single gene mutations, chromosomal rearrangements, mitotic cross-overs and cytoplasmic DNA changes (Evans et al., 1984). Tissue culture can therefore provide breeders with another avenue of variation to explore. Many agronomic traits, including resistance to specific herbicides, are expressed at the cellular level. The ability to select specific mutants at the cellular level should allow breeders the opportunity to find the variation necessary for improvement. This source of variation is particularly important if there is a narrow base of germplasm for a specific crop species which results in reduced whole plant variation. These cellular methods require an efficient plant regeneration system if they are to be effectively utilized.

Classical research in selecting for herbicide tolerance was reported in 1978 by Chaleff and Parsons. They conducted this work on tobacco (Nicotiana tabacum L.) with the herbicide picloram [4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid]. The objectives of their study were to select tolerant mutants that would be agronomically useful as well as to determine the mode of action of the herbicide.

Suspension cultures were filtered through cheesecloth and cultures were plated onto medium containing 500 uM

picloram. After 1-2 months on the selection media resistant callus was isolated. Callus that continued to grow on a second passage through the picloram medium were transferred to shoot-induction medium.

Seven cell lines were isolated and shown to be tolerant to picloram. One cell line could not be regenerated. The regenerated plants of the other six lines were induced to form callus for a second cycle to determine if the callus of regenerated plants was also tolerant. Four lines proved to be tolerant. Crosses made between plants derived from the four tolerant cell lines indicated that the tolerance was inherited as a single dominant allele in three of the cell lines. The fourth mutant line was also controlled by a single gene but this one was semidominant. Further studies showed that the four mutations defined three distinct linkage groups (Chaleff, 1980).

Chaleff and Ray (1984) did similar research on tobacco but this time screening for tolerance to the herbicides chlorsulfuron [2-chloro-N-[[(4-methoxy-6-methyl-1, 3,5-triazin-2-yl)amino] carbonyl] benzenesulfonamide] and sulfometuron methyl [2-[[[[[4,6-dimethyl-2-pyrimidinyl] amino]carbonyl] amino] sulfonyl] benzoic acid]. They were able to select several mutants tolerant to the herbicides using the methods described above. Regeneration and sexual crosses proved that resistance was inherited as a single dominant or semidominant mutation and that there were two unlinked genetic loci.

The selection of herbicide tolerant variants in cell culture is of importance not only to the plant breeder but also to the biochemist. Cell cultures provide a unique opportunity to study the modes of action of herbicides. Chaleff and Mauvais (1984) were able to determine that chlorsulfuron and sulfometuron methyl inhibit acetolactate synthase, the first enzyme specific to the amino acid biosynthetic pathway. Chaleff and co-workers have provided examples of complete research projects; they have selected mutants in culture, regenerated plants, performed sexual crosses to determine the method of inheritance, and conducted biochemical studies to ascertain the mode of herbicide action. As a result, these systems are often used as model projects for other researchers studying the feasibility of developing herbicide tolerance via cell cultures.

Selection for Tolerance to 2,4-D

Swanson and Tomes (1979, 1983) isolated calles of birdsfoot trefoil that was capable of growth on high levels of 2,4-D and proved that it was considerably more tolerant than control calles. Four different calles lines were regenerated. Three of these lines produced plants with tolerance to 2,4-D. The regenerated plants did not, however, express as much tolerance as the tolerant control.

which was selected by 5 cycles of recurrent selection at the whole plant level. Regenerated plants were inbred and had reduced levels of pollen viability. The progeny from an intercross of the tolerant plants were susceptible to 2,4-D and the authors suggest that this was due to inbreeding depression. A topcross to a susceptible genotype did increase the tolerance of the progeny of the susceptible line suggesting a genetic basis for the tolerance.

Deo (1979) were able to select a stable variant cell line of tobacco that was resistant to 2,4-D. This tolerance was maintained under non-selective, standard growth conditions. Regenerated plants did not express the 2,4-D tolerance but callus re-induced from the regenerated plants retained the 2,4-D tolerance. These clones possessed cross-resistance to the auxins IAA, NAA and picloram (Nakamura et al., 1985).

Gressel (1979) treated suspension cultures of carrot (Daucus carota L. var. sativa DC.) with 1 g L^{-1} of 2,4-D for three hours, followed by rinsing with water, then plated cells in agar medium. This procedure was repeated several times. Gressel was able to isolate a strain of cells which could withstand a concentration of 2,4-D 100 times that of the unselected control. After one year of growing the cells without 2,4-D, these cell lines still retained their tolerance, which suggests a stable genetic

change had occurred. The author has not reported regeneration of tolerant plants.

Zenk (1974) used haploids of Nicotiana sylvestris Spegazzini & Comes (n=12) to select for 2,4-D tolerance in suspension cultures. Gradual increases in 2,4-D concentration over 1.5 years led to the selection of a strain tolerant to concentrations of 2,4-D of up to 10^{-3} M. No regeneration was reported.

Oswald et al. (1977) conducted experiments on 2,4-D tolerance in white clover suspension cultures. They found that a five day pretreatment with either 2,4-D, 2,4,5-T [(2,4,5-trichlorophenoxy) acetic acid], or 2,4-DB [4-(2,4-dichlorophenoxy) butanoic acid] increased cell tolerance levels. Tolerance was transmitted to succeeding cell generations but plant regeneration was not achieved at the time of the report.

Some research reports indicate that tolerance is lost after subculturing in the absence of the selection pressure. Widholm (1977) transferred a 2,4-D tolerant suspension culture of carrot cells to a medium that did not contain 2,4-D. When put back on the selective medium, the tolerant cell line no longer expressed its tolerance to 2,4-D. This suggested that the initial tolerance may have been due to the induction of enzymatic systems that had the capability of degrading 2,4-D. Once the inducer was removed, the enzymes were not being produced and the herbicide tolerance was lost.

Selection for Tolerance to Other Herbicides

Thomas and Pratt (1982) used techniques similar to the ones described by Chaleff and Parsons (1978) to isolate tomato clones tolerant to paraquat. Nineteen such clones were isolated and plants were regenerated from nine of these. Sexual crosses showed that a single dominant nuclear mutation was responsible for three of the tolerant phenotypes. Only one regenerated plant, however, expressed slightly increased levels of tolerance to paraquat. The cellular cultures of the progeny of regenerated plants retained tolerance. This is an example of the trait specifically selected for in cell culture not being fully expressed at the whole plant level; a significant obstacle to the success of many variant selection systems.

The methods described by Chaleff and Parsons (1978) are suitable for herbicides that are capable of affecting callus tissue. Not all herbicides are toxic to cells in culture. The mode of action of some herbicides is that of photosynthetic inhibition, and would have little or no effect on non-photosynthetic callus tissue. Ellis (1978), working with non-photosynthetic cell suspension cultures of tomato, found that there was no difference between four cultivars for tolerance to the photosynthetic inhibitor, metribuzin. These same four cultivars, however, expressed differential sensitivity at the whole plant level. Ellis

(1978) did show that growth levels of cell cultures could be reduced if very high rates (> 100 ppm) of metribuzin were applied to the cultures and suggested that it might be possible to select for higher rates of metabolism. When first beginning selection work at the cellular level it is important to try to establish an in vivo to in vitro phenotypic-response correlation. This means that the response to a selection pressure of the callus tissue should be similar to the response seen at the whole plant level. If this correlation was established there would be an increased probability that the selected trait would be expressed in regenerated plants (Grassel et al., 1978).

Radin and Carlson (1978) devised a unique technique that enabled them to select for tolerance to herbicides that affect photosynthesis. The herbicides chosen were bentazon [3-(1-methylethyl)-(1H)-2, 1,3-benzothiadiazin-4(3H)-one 2,2-dioxide) and phenmedipham [3-[(methoxycarbony) amino] phenyl (3-methylphenyl) carbamate]. They sprayed the herbicides on tobacco plants that had been mutagenized and isolated green islands on otherwise yellow, dying leaves. These islands were regenerated in culture and subsequent plants were analyzed via sexual crosses. Twenty-one percent of the bentazon-sprayed regenerates and 13% of the phenmedipham-sprayed regenerates retained their tolerance. In sexual crosses, all F_1 plants were herbicide sensitive

but the F_2 plants provided the necessary segregation to help delineate the sexual processes that were involved. Herbicide tolerance appeared in eight of the 15 bentazon lines and two of the seven phenmedipham lines, indicating that the tolerance was controlled by a single recessive gene. Further tests, however, indicated that more than one allele may have been responsible for the tolerance.

Another method of selecting for mutants tolerant to photosynthetic-inhibiting herbicides in cell cultures has been recently reported (Wang and Collins, 1985). This work utilized photomixotrophic cell cultures of tobacco to isolate clones tolerant to atrazine. The cell cultures were capable of synthesizing chlorophyll which enabled the atrazine to become active in the green tissue and therefore was toxic to the cells. Thirty-seven tolerant plants were regenerated. Crosses and selfs were made and the progeny are currently being evaluated.

There has been some success reported recently on corn with a broad-spectrum herbicide developed by American Cyanamid Company called imazaquin [2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H- imidazol-2-yl]-3-quinolinecarboxylic acid] (Anderson et al., 1984). Corn cell lines were found that expressed greater than a 100-fold increase in tolerance to the herbicide. Plants were regenerated on medium which also contained normally toxic levels of imazaquin. Genetic studies of R
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(regenerated first generation plants) progeny indicated that the tolerance was controlled by a single dominant gene.

Summary and Conclusions

The development of a tolerant cell is only an initial step. The desired outcome of any selection program should be development of a cultivar of tolerant plants that have agronomic potential. Many reports end by stating that regeneration is now in progress and plants will be evaluated for tolerance. Cell to plant regeneration systems research must be expanded. Homer LeBaron, coeditor of Herbicide Resistance in Plants (1982) calls for a more active interfacing among the chemical industries, the seed companies, and the plant breeders to accomplish these goals (LeBaron, 1984). He suggests that the logical source for this type of work is within the seed companies. Currently, much of the work is being conducted by public universities. The seed companies, however, should see the economic potential of these tolerant varieties. A high yielding cultivar of soybean, for instance, that was tolerant to a broad-spectrum herbicide would have tremendous potential in the marketplace, particularly if the tolerance was inherited qualitatively. From the complete research works that have been reported to

date, it is apparent that if tolerance is to be gained, it will be for traits that are controlled qualitatively. There are definite obstacles to overcome in selecting for herbicide tolerance at the cellular level. If variability for a certain trait is not present at the whole plant level, then selecting at the cellular level becomes an alternative.

CHAPTER III

RECURRENT HALF-SIB FAMILY SELECTION FOR 2,4-D TOLERANCE IN RED CLOVER

Introduction

There has been debate concerning the relative effectiveness of conventional and molecular breeding techniques and their respective roles in crop improvement. Conventional breeding methodologies are well-known and few can argue with the successes of these traditional programs (Borlaug, 1983; Sprague et al., 1980). Proponents of the biotechnological methods, however, have criticized conventional breeding as being labor- and land-intensive (Grassel and Segal, 1978; Crocomo and Ochoa-Alejo, 1983). Others consider conventional breeding to be an inefficient and time-consuming process that has resulted in a reduction in crop variability (Hughes, 1984; Gressel, 1979).

Cellular selection has been promoted as a tool that can reduce the time and space involved in the breeding processes by making selections manageable and more efficient (Duncan and Widholm, 1986). In vitro culture systems have also been regarded as an alternative procedure for increasing the available variability for a particular crop species (Larkin and Scowcroft, 1981).

Conventional breeders will argue that regenerated plants, selected in vitro, still must undergo traditional plant breeding methods and evaluations so that they can be placed in suitable genetic backgrounds. Bingham (1983) further argues that in vitro selection will be of little value when breeding directly for multiple gene traits such as yield, which comprises a large percentage of today's crop improvement efforts.

Today's plant breeders must be aware of these factors when embarking upon a project in crop improvement. It is the breeders position to evaluate and compare new and old technologies and to incorporate the more appropriate ones into a breeding project. The overall objective of our research was to take a particular trait, in this instance, 2,4-D [(2,4-dichlorophenoxy) acetic acid] tolerance in red clover (Trifolium pratense L.), and select for it in vivo and in vitro. This not only afforded us an avenue of comparison, but also enabled us to learn more about the respective programs and to discover their advantages and disadvantages. This would allow the opportunity to determine how each method can be productively blended into the overall forage legume breeding project at the University of Florida.

The advancement of biotechnological techniques has resulted in an increased awareness in the development of herbicide-tolerant crop cultivars. Most of the recent research has involved cellular selection techniques (Meredith and Carlson, 1982). This work has usually been conducted at the expense of the whole plant selection methods, which in certain instances, may provide better results in terms of pure crop improvement. Surprisingly, there has been little in vivo development of herbicide-tolerant crop cultivars (Faulkner, 1982). Gressel (1979) feels that field herbicide screening is inappropriate because the selection pressure imposed in the field can rarely be made to exceed 90 to 95% kill and that the remaining plants would likely be escapes. He suggests that increasing the herbicide rate would kill the truly tolerant plants and indicates this to be a reason for the few reports of successful selection for herbicide tolerance in the field.

Phenotypic recurrent selection was used by Devine et al. (1975) to develop a line of birdsfoot trefoil (Lotus corniculatus L.) tolerant of 2,4-D. Progeny testing allowed for the selection of 34 clones from an original population of 75. These 34 clones were intercrossed, progeny were established in the field and sprayed with 1.7 to 2.4 kg ha^{-1} 2,4-D. Selections were based on ratings of regrowth. After five such cycles, evaluation tests

determined that recurrent selection was effective in increasing plant tolerance levels. Survival percentage, dry weight, plant nitrogen composition and surface wax accumulation were higher in the tolerant strain than in the susceptible genotype.

Faulkner (1975) developed and registered a paraquat (1,1'dimethyl-4,4'-bipyridinium ion) tolerant line ('Causeway') of Lolium perenne L., also by recurrent selection. Faulkner (1978) also selected L. perenne lines that were tolerant to the herbicide dalapon (2,2-dichloropropanoic acid) and possessed good agronomic characteristics. Recurrent selection was also used by Wright and Faulkner (1982) in selecting for glyphosate (N-(phosphonomethyl) glycine) tolerance in L. perenne. The populations selected were 50 to 67% more tolerant than the controls.

The specific objective of our research was to use phenotypic recurrent selection to develop a 2,4-D tolerant red clover population. In vitro selection techniques are discussed in the following chapter and the more salient comparisons between the two breeding methods will be addressed therein.

Materials and Methods

Selection of 2,4-D Tolerant Plants

The originally planned selection procedure was phenotypic recurrent mass selection. On 24 Feb. 1985, 20,000 seed from 3 sources ('Kenstar', 'Nolin's', and a local breeding population called QC5) were broadcast seeded into metal flats containing an equal mixture of Arredondo fine sand [loamy, siliceous, hyperthermic, Grossarenic Paleudult (5% silt, 2% clay, 92% sand, 1% organic matter, pH 6.1)] and Metro Mix Growing Medium 500® [1] (W. R. Grace and Co., Cambridge, MA 02140). When the seedlings possessed 3 trifoliate leaves (100 mm tall on 29 Mar. 1985) they were sprayed with 2,4-D. The herbicide was mixed with water and sprayed with a CO_2 backpack sprayer at a volume of 187 L ha^{-1}. The applications were made with size 11003 nozzle tips at 172 kPa while walking past the flats at 1.4 m s^{-1}. Plants were sprayed with 1.1 kg a.i. ha^{-1} 2,4-D. Due to low selection pressure the plants were resprayed using similar techniques on 19 Apr. 1985. On 19 May 1985, 385 plants were selected for intercrossing. Selection criteria were based on survival and regrowth potential. These plants were transplanted in the field and

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[1] Mention of a trade name is only for the purpose of identification and does not constitute a warranty to the exclusion of other suitable substitute products.

covered with a cage. A hive of honey bees (Apis millifera L.) was placed in the cage to serve as pollinators. Seed was harvested separately from 63 individual plants on 18 Aug. 1985.

After the initial cycle, the breeding procedure was changed to a recurrent half-sib family selection program. The remaining cycles of selection were conducted using the following procedures. Dates, number of plants tested, and selection intensities for each cycle are shown in Table 3-1 and 3-2 (For clarity, cycle 0 seed refers to the starting population; cycle 0 seed undergoes selection cycle 1, selected plants are then intercrossed, thereby creating cycle 1 seed). Seed from half-sib families in each cycle were screened for 2,4-D tolerance in a 3-replicate randomized complete block evaluation test. Seeds of each individual half-sib family were germinated in petri dishes and seedlings were transplanted into individual cells of #200 Todd planter flats® [1] (Speedling Incorporated, P. O. Box 220, Sun City, FL 33586). Each cell held approximately 100 cm^3 of an equal mixture of Arredondo fine sand and Metro Mix 500. There were 6 plants of each family per replication. At the same time, 10 extra plants from each family were planted as remnants and were not considered to

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[1] Mention of a trade name is only for the purpose of identification and does not constitute a warranty to the exclusion of other suitable substitute products.

Table 3-1. Dates of events during a recurrent selection program to increase levels of 2,4-D tolerance in red clover.

Cycle	Planted	Sprayed	Rated	Harvested
1	2/24/85	4/19/85	5/22/85	8/18/85
2	8/23/85	9/18/85	10/02/85	12/27/85
3	1/14/86	2/07/86	2/28/86	6/12/86
4	7/02/86	7/23/86	8/07/86	3/13/87 +

+ Numerous plants from this cycle originally died in the field due to environmental stresses. More plants were grown at a later date to replace those plants that died. An earlier partial harvest took place on 12/20/86.

Table 3-2. Number of plants and families tested, number of families selected and intercrossed, and selection intensities per cycle in a recurrent selection program to increase levels of 2,4-D tolerance in red clover.

Cycle	Plants Tested	Families Tested	Families Selected	Plants Crossed	Selection Intensity
	----------	no.	----------	----------	----%----
1	20,000	----	63	63	0.3
2	1,134	63	21	210	33.3
3	3,456	192	25	302	13.0
4	4,968	276	22	220	7.9

be a part of the evaluation experiment or sprayed with 2,4-D. Approximately 3 weeks after planting, plants were sprayed with 1.1 kg $s.i.\ ha^{-1}$ 2,4-D using methods described earlier. Three weeks after spraying, individual plants were removed from the cells and roots were rinsed with water to remove the soil. Each plant was examined and assessed a visual rating on 1-5 scale (5 = dead) based on the amount of stunting, stem curvature and root damage. Mean family ratings were calculated and used to make family selections. The ten remnant plants from each selected family, which had not been sprayed, were taken to the field, completely randomized, and intercrossed using honey bees as pollinators. Depending upon the season of the year, plant lights were utilized to extend the photoperiod to induce red clover plants to flower. These procedures enabled us to complete 2 cycles of selection each year.

Data were taken on an individual plant basis to obtain an estimate of within half-sib family variance. Family selections were based on family means, so narrow-sense heritability (h^2) was calculated using the phenotypic family mean as the selection unit (Nguyen and Sleper, 1983). Therefore:

$$h^2 = \frac{V_f^2}{V^2/r + V^2/rn + V_w^2/rn}$$

where r=number of replications, n= number of individual plants per plot; and V_w^2 = variance among individual plants within plots. Realized heritabilities were calculated by dividing the gain observed in the following cycle by the selection differential. Expected gain was derived from the product of the selection differential and the narrow-sense heritability.

Theoretical coefficients of inbreeding (approximate F-values) can be calculated using this formula (P. Cornelius, 1986, personal communication):

$$F = 1 - \left(1 - \frac{1}{2Ne}\right)^g$$

where g = cycle number; and

$$Ne = \frac{(4)(n_1)(n_1 + n_2)}{(4)(n_1) + (n_2)}$$

with n_1 = the number of individuals contributing to the next generation as female parents; $n_1 + n_2$ = the total population size or the total number of individuals who can contribute offspring to the next generation as male parents; therefore $n_2 = (n_1 + n_2) - n_1$. Since Ne differed for each generation, this formula was used in place of the above formula:

$$F = 1 - \left(1 - \frac{1}{2Nh}\right)^g$$

where Nh is the harmonic mean of the Ne values of the generations up to, but not including, generation g.

Evaluation of All Cycles

One greenhouse and one field experiment were conducted to evaluate the progress made from 4 cycles of the recurrent selection program to increase levels of 2,4-D tolerance in red clover. Both experiments included the following entries: cycle 0 (a bulk seed mixture of Kanstar, Nolin's seed QC5), cycle 1, cycle 2, cycle 3, and cycle 4. The bulks from the individual cycles were obtained by mixing equal proportions from all half-sib families in that particular cycle.

Field evaluation of all cycles

Seed of the 5 cycles were germinated in petri dishes and planted in #200 Todd planter flats containing an equal mixture of Arredondo fine sand and Metro Mix 500 on 9 Jan. 1987. On 11 March, 1987, the plants were transplanted to a field at the University of Florida Beef Research Unit, north of Gainesville. The Wachula soil (sandy, siliceous, hyperthermic, Ultic Haplaquod) and covering vegetation was prepared by a burn down application of glyphosate and an application of 450 kg ha^{-1} 0-10-20 ($N-P_2O_5-K_2O$), two days prior to planting.

Six plants of each cycle were planted 0.5 m apart within rows that were 0.5 m apart. The design was a 4-replicate split-plot with rates of 2,4-D as main plots and cycles of red clover as subplots. The rates of 2,4-D

used were 0, 0.56, 1.12, 1.7, 2.2, and 4.5 kg a.i. ha^{-1}. On 3 Apr. 1987, when the plants were approximately 25 cm tall, they were sprayed with the various rates of 2,4-D using a CO_2 backpack sprayer (187 L ha^{-1} water, 172 kPa, walking speed = 1.4 m s^{-1}). Individual plants were visually rated for 2,4-D injury using the 1-5 (5=dead) rating scale 3 weeks after spray application.

Greenhouse evaluation of all cycles

Seed of each population were germinated in petri dishes on 10 March, 1987, and transplanted into #200 Todd planter flats containing an equal mixture of Arrendondo fine sand and Metro Mix 500. The split-plot design had 4 replications, main plots as rates of 2,4-D and subplots as entries of red clover. On 7 Apr. 1987, seedlings were sprayed with the same rates and methods used in the field experiment. Three weeks after treatment, plants are visually rated for 2,4-D injury using the 1-5 rating system.

For both experiments, analysis of variance was used initially to test model significance. Regression procedures were used to examine relationships between mean visual ratings and cycle number. Best fitting linear equations were chosen according to the significance of F-values, R^2 values and the parameter estimates.

Results and Discussion

Individual Cycle Evaluations

The original intention of our research was to use recurrent mass selection. During the first cycle of selection, however, sprayed plants were slow to outgrow the 2,4-D damage. As a consequence, only 63 plants out of 385 originally selected survived to set seed. These survivors were low in vigor and did not set many seed. It became obvious that alterations in the design would have to be made to reach the objective of 2 cycles of selection per year. The recurrent half-sib family selection design proved to be very suitable. The use of non-sprayed remnant plants, increased the vigor of the selections which also increased seed yield. By planting the remnant plants at the same time as the actual evaluation test, 6 to 8 weeks of time were saved. By the time the family selections were made, these remnant plants were ready to be transplanted in the field for intercrossing. The use of lights to extend the photoperiod allowed for the completion of 2 cycles of selection per year. This was beneficial because short cycle times are very conducive to the use of a recurrent selection program. Also, because half-sib families were used, the expected gain per cycle was higher than gains expected when using recurrent mass selection.

As for the disadvantages of this method, it was very labor-intensive. Each seedling was planted by hand to assure a full stand for a statistically balanced model and the pedigree of each plant was maintained. Also, because we planted remnant plants for every family, not just the selected ones, extra work was required. Approximately 90% of these remnants were never used.

As a result of the numerous deaths in cycle 1, the selection pressure was a very intense 0.3%. This was both beneficial and detrimental to the program. The high pressure resulted in a substantial gain of 0.75 on the 1-5 rating scale (Table 3-3) but also left only 63 half-sib families for the next cycle. Due to the effects of inbreeding depression it was necessary to increase the number of families tested and consequently 33% were selected in cycle 2. The trend after that was to test more families and keep the number of families selected about the same, thereby increasing the selection intensity. As progress becomes more difficult to realize in the later cycles of a recurrent selection program, one of the methods of compensation is to increase the selection intensity

Using the individual evaluation experiments as a gauge of the progress made, one would have to conclude that the program has been successful in increasing the levels of 2,4-D tolerance. The mean population rating has decreased 39%, an average decrease of 13% per cycle (Table 3-3). The

Table 3-3. Means, variances, actual gains, expected gains, selection differentials, narrow-sense heritabilities, realized heritabilities, and approximate percent inbreeding values calculated from individual cycle evaluation tests during a recurrent selection program to increase levels of 2,4-D tolerance in red clover.

	Mean	Total Variance	Next Cycle	Expected Gain	Select. Differ.	Narrow Herit.	Realiz. Herit.	Approx. Inb. %
Cycle 0	4.2	---	0.75	---	---	---	---	0.0
Cycle 1	3.45	0.81	0.34	0.49	0.84	0.59	0.40	0.008
Cycle 2	3.11	0.94	0.53	0.51	1.05	0.49	0.50	0.015
Cycle 3	2.58	0.96	---	0.44	0.83	0.53	---	0.02

Genetic parameters were not estimable in this cycle because selections were based on survival and no data were taken.

* Means based on a 1-5 visual rating system with 5 = dead plants.

total variance and inbreeding depression values indicate that variability has not been reduced across the cycles and inbreeding is at a minimum. Heritability estimates have been high (49-59%) and fairly constant over the cycles of selection. Realized heritabilities were comparable to the narrow-sense heritabilities and fairly constant, ranging from 40 to 50%. Gains observed averaged 0.54 units per cycle and were comparable to expected gains. All data indicate that the progress has not peaked and further progress should be possible. The cycle 3 population, with a rating of 2.5%, is quite tolerant. Plants from selected families showed very few symptoms of 2,4-D injury even 2 weeks after spray application. It is interesting to note that all plants showed similar initial symptoms of 2,4-D injury immediately after spraying. However, after 36 to 48 h, the most tolerant plants started to recover and rapidly outgrew the damage. Susceptible plants were not capable of regrowth and eventually died. These are indications of a metabolic type of 2,4-D tolerance in red clover. Future research efforts may be aimed at finding the mechanisms for tolerance.

Throughout the cycles of selection, attempts were made to maintain the integrity of the visual rating system. Steps were taken to standardize conditions but this was difficult to do as the individual cycle evaluations were conducted over 2 years at different times of the year and

the characteristics of the plant populations fluctuated. The family frequency distributions have been shifted toward the lower end of the scale (Table 3-4). In selection cycle 4 there were very few families rating ≥ 4 or greater, while the percentage of families rating less than 2 was significantly increased since selection cycle 2.

Evaluation of All Cycles

Both the greenhouse and field evaluation experiments further illustrate the increased levels of 2,4-D tolerance gained by 4 cycles of the recurrent half-sib family selection program (Table 3-5). At each of the rates, cycle 4 proved to be the most tolerant or equal to the most tolerant cycle tested. The mean rating of cycle 4 has been decreased an average of 28.5% over cycle 0, over all 5 rates of 2,4-D. At the selection rate (1.12 kg a.i./ha), cycle 4 was 38% and 32% more tolerant than cycle 0 in the greenhouse and field experiments, respectively. This is not as great as the 39% gain showed by the individual evaluation experiments after only 3 cycles of selection (note: the individual half-sib families of cycle 4 have yet to be evaluated).

Over the five rates of 2,4-D, the greatest gain observed was after the first cycle of selection (0.5 units) This was also true in the individual evaluation experiments. Very little gain was observed in the

Table 3-4. Half-sib family mean rating frequency distributions over 4 cycles of a recurrent selection program designed to increase levels of 2,4-D tolerance in red clover.

Family Mean Rating +	Cycle of Selection		
	Two	Three	Four
	----percentage of families----		
1.0 - 1.59	0.0	0.0	1.0
1.6 - 2.09	1.5	1.5	12.3
2.1 - 2.59	4.7	13.0	35.8
2.6 - 3.09	19.0	33.8	38.7
3.1 - 3.59	39.6	35.4	8.6
3.6 - 4.09	22.2	13.5	2.9
4.1 - 4.59	7.9	2.1	0.4
4.6 - 5.0	4.8	0.5	0.0

+ Mean ratings are based on a 1-5 visual rating system (5=dead plant).

Table 3-5. Mean visual 2,4-D injury ratings over 5 cycles of a recurrent selection program designed to increase levels of 2,4-D tolerance in red clover. Visual ratings were taken 3 weeks after red clover plants were sprayed with 5 different rates of 2,4-D in both the field (FD) and greenhouse (GH) evaluation experiments.

	Rate of 2,4-D (kg a.i./ha)									
	0.56		1.12		1.7		2.2		4.5	
	GH	FD	GH	FD	GH	FD	GH	FD	GH	FD
	Mean visual injury rating*									
Cycle 0	2.5 ab	3.4 a	3.7 ab	4.0 a	4.4 a	4.1 ab	4.6 a	4.6 a	4.8 a	4.9 a
Cycle 1	2.2 bc	2.3 c	3.4 bc	3.7 a	3.5 b	3.7 bc	4.1 b	4.0 b	4.8 a	4.4 b
Cycle 2	1.7 c	2.6 b	3.0 c	3.0 b	3.2 bc	3.3 cd	3.9 b	3.2 c	4.5 ab	4.2 b
Cycle 3	1.8 c	2.3 c	3.1 c	2.8 b	3.0 cd	3.1 d	3.8 b	3.8 b	4.7 a	3.9 c
Cycle 4	1.4 d	2.2 c	2.3 d	2.7 b	2.8 d	3.1 d	3.9 b	3.3 c	4.5 ab	3.6 c

Mean visual ratings are based on a 1-5 scale (5=dead) Values are the means of 5 observations pooled from 4 replications.

* Means in the same column followed by the same letter are not significantly different according to Duncan's new multiple range test (P=0.05).

third cycle of selection. In fact, cycle 2 was equal to or better than cycle 3 in both the field and greenhouse evaluations. The gain between cycle 3 and 4, however, was better and averaged 0.25 units. All experiments demonstrate the trend that the gain per cycle is decreasing as more cycles of selection are conducted.

For each rate in each experiment there was a significant linear relationship between the mean visual rating and the cycle of selection (Figure 3-1 to 3-5). These figures would indicate that future progress in increasing levels of 2,4-D tolerance should be possible. It is necessary to further increase these tolerance levels because they are not high enough to be practical in the field at this time. At the current pace of 0.25 units of gain per cycle at least 4 more cycles of selection would be needed to obtain a desirable tolerance level of below 1.5 (assuming cycle 4 rates 2.5). An alternative would be to alter the breeding design to increase the gain per cycle. Plants in cycle 4 are now tolerant enough to 2,4-D that the use of remnant plants can be abandoned. Gains could be increased by selecting only the best plants out of the best families after a spray application. This alteration would also serve to reduce labor and other capital inputs.

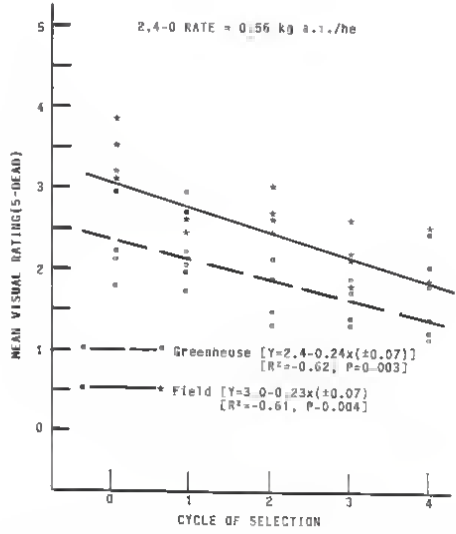

Figure 3-1. Mean visual 2,4-D injury ratings over four cycles of a recurrent selection program designed to increase levels of 2,4-D tolerance in red clover. Visual ratings were taken three weeks after red clover plants were sprayed with 0.56 kg a.i. 2,4-D/ha in both the field and greenhouse evaluation experiments.

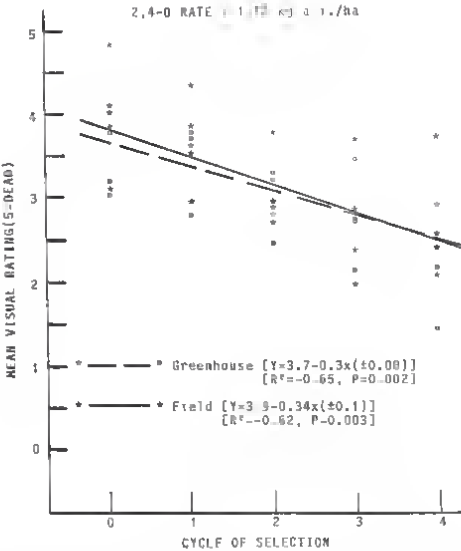

Figure 3-2. Mean visual 2,4-D injury ratings over four cycles of a recurrent selection program designed to increase levels of 2,4-D tolerance in red clover. Visual ratings were taken three weeks after red clover plants were sprayed with 1.12 kg a.i. 2,4-D/ha in both the field and greenhouse evaluation experiments.

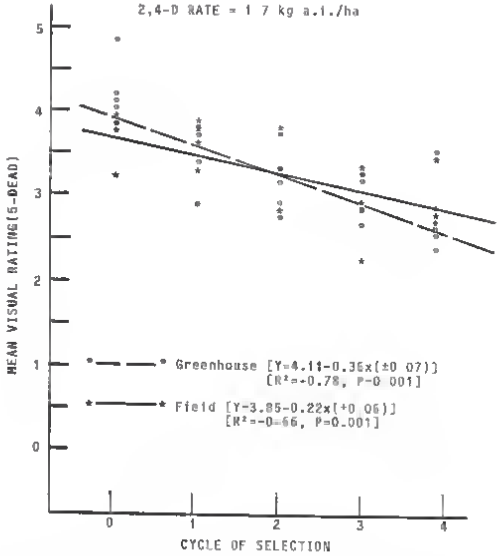

Figure 3-3. Mean visual 2,4-D injury ratings over four cycles of a recurrent selection program designed to increase levels of 2,4-D tolerance in red clover. Visual ratings were taken three weeks after red clover plants were sprayed with 1.7 kg a.i. 2,4-D/ha in both the field and greenhouse evaluation experiments.

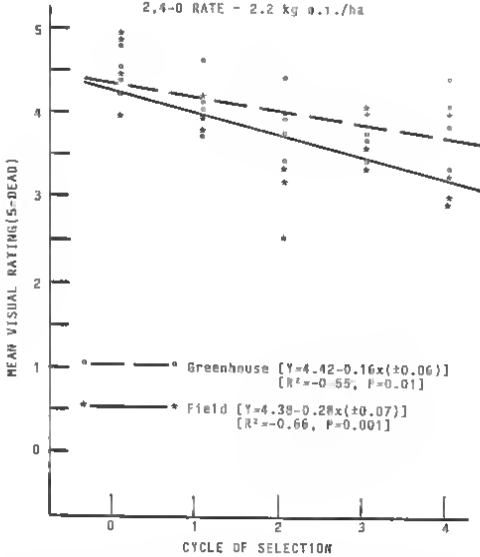

Figure 3-4. Mean visual 2,4-D injury ratings over four cycles of a recurrent selection program designed to increase levels of 2,4-D tolerance in red clover. Visual ratings were taken three weeks after red clover plants were sprayed with 2.2 kg a.i. 2,4-D/ha in both the field and greenhouse evaluation experiments.

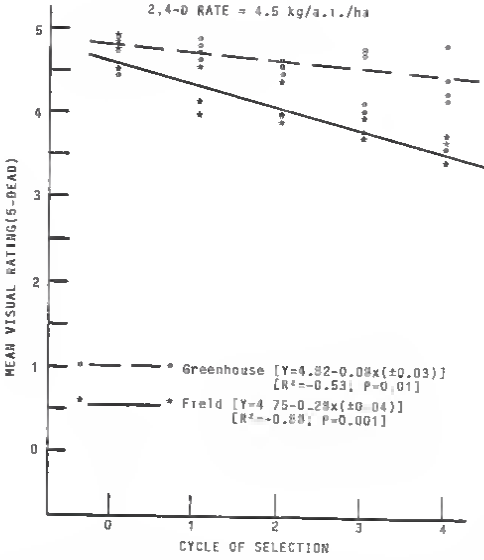

Figure 3-5. Mean visual 2,4-D injury ratings over four cycles of a recurrent selection program designed to increase levels of 2,4-D tolerance in red clover. Visual ratings were taken three weeks after red clover plants were sprayed with 4.5 kg a.i. 2,4-D/ha in both the field and greenhouse evaluation experiments.

In summary, four cycles of recurrent selection have increased levels of 2,4-D tolerance approximately 35% in red clover. The narrow-sense heritability of the tolerance trait was high, averaging nearly 50% and inbreeding depression was kept to a minimum. The significant linear relationship between mean visual ratings and cycle of selection indicated that future progress should be possible. Several more cycles of selection must be conducted to obtain tolerance levels high enough for use in a pasture situation. It is suggested that the breeding design be altered to facilitate greater gains per cycle. The agronomic potential of the 2,4-D tolerant population must also be further investigated to ascertain that yield levels have not significantly decreased. If yields are not lowered and other agronomic variables are stable, then the use of recurrent selection to develop herbicide tolerant cultivars should prove to be a viable alternative to either using in vitro methods or formulating selective herbicides.

CHAPTER IV

IN VITRO SELECTION FOR 2,4-D TOLERANCE IN RED CLOVER

Introduction

There has been a concomitant increase in interest in herbicide tolerance with the advancement of biotechnological methodologies. Herbicide tolerance has been identified as one of the traits that is well suited to in vitro selection schemes. Millions of potentially different cells can be quickly and efficiently screened due to the uniformity of the culture environment. It is also believed that cell cultures can provide new sources of crop variability and produce plants with alternate forms of herbicide tolerance (Hughes, 1984; Meredith and Carlson, 1982). In addition, cell cultures provide a unique opportunity to study biochemical and physiological relationships between crop and herbicide (Chaleff and Mauvais, 1984).

In vitro selection for 2,4-D [(2,4-dichlorophenoxy) acetic acid] tolerance has been attempted by several researchers with little more than modest success. This can possibly be attributed to the complex nature of the mode of action of 2,4-D, which is poorly understood, and the seemingly complex genetics that are required to obtain

tolerances. The more successful cellular selections to date have involved tolerances that are under the control of qualitative genetic systems, such as that shown for picloram (4-amino-3,5,6-trichloro-2- pyridinecarboxylic acid) and tobacco (Nicotiana tabacum L.) (Chaleff and Parsons, 1978). Also, since 2,4-D has auxin-like properties and is used as an auxin in many tissue culture systems, this may result in undefined interactions within the culturing environment. As an auxin, 2,4-D is believed to induce changes in callus tissue that can be detrimental and alter regenerative potential. These factors may cumulatively be responsible for the difficulties involved in selecting for 2,4-D tolerance in vitro.

Swanson and Tomes (1979, 1983) isolated callus of birdsfoot trefoil (Lotus corniculatus L.) that was capable of growth on high levels of 2,4-D. Three callus lines produced regenerated plants with low levels of tolerance to 2,4-D. The regenerated plants did not express as much tolerance as the tolerant control, which was selected by five cycles of recurrent selection at the whole plant level. Ono (1979) was able to select a stable variant cell line of tobacco that was resistant to 2,4-D. Regenerated plants did not express the 2,4-D tolerance but callus re-induced from the regenerated plants retained the 2,4-D tolerance. These clones possessed cross-tolerance to the auxins IAA, NAA and picloram (Nakamura et al., 1985).

Greesel (1975) was able to isolate a strain of carrot (Daucus carota L. var. sativa DC.) cells which could withstand a concentration of 2,4-D 100 times that of the unselected control. After one year of growing the cells in the absence of 2,4-D, these cell lines still retained their tolerance, which suggested that a stable genetic change had occurred. Regeneration of tolerant lines was not reported. Oswald et al. (1977) conducted experiments on 2,4-D tolerance in white clover (Trifolium repens L.) suspension cultures. They found that a five day pretreatment with either 2,4-D, 2,4,5-T [(2,4,5-trichlorophenoxy) acetic acid] or 2,4-DB [4-(2,4-dichlorophenoxy) butanoic acid] increased cell tolerance levels to these herbicides. Tolerance was transmitted to succeeding cell generations but plant regeneration was not achieved at the time of the report. Some research reports indicate that tolerance is lost after subculturing in the absence of the selection agent. Widholm (1977) transferred a 2,4-D tolerant suspension culture of carrot cells to a medium that did not contain 2,4-D. When placed back on the selective medium, the tolerant cell line no longer expressed its tolerance to 2,4-D. This suggested that the initial tolerance was not genetically based.

The feasibility of an in vitro selection system increases if a correlated in vivo to in vitro response

exists (Gressel et al., 1978). It should be determined if tolerant whole plants are also tolerant in vitro, as shown with 2,4-D and birdefoot trefoil (Swanson and Tomes, 1980). This correlation would tentatively establish that a 2,4-D metabolic pathway does exist within the plant species of question. This would be in contrast to tolerance based on morphological traits at the whole plant level which would not transfer to in vitro systems. If this metabolic pathway is genetically based, then it is theoretically possible to select callus cultures with a similar mechanism of tolerance. While not definitive, this correlation may provide sufficient justification to use cell cultures to screen for 2,4-D tolerance.

The objectives of this research were 1) to determine if there was a correlated in vivo to in vitro response to 2,4-D within red clover (T. pratense L.), 2) to screen genotypes for regenerative ability, 3) to select 2,4-D tolerant cell lines from regenerating genotypes, 4) to define the level of 2,4-D tolerance of selected callus lines, 5) to attempt to regenerate plants from the 2,4-D tolerant callus lines, and 6) to compare in vitro and in vivo methods of selecting for 2,4-D tolerance in red clover. The in vivo method of selection was discussed in the previous chapter.

Materials and Methods

Correlated Response to 2,4-D

Eight red clover genotypes chosen from the third cycle of a recurrent selection program for 2,4-D tolerance, were utilized in a greenhouse and a tissue culture experiment to determine the relative 2,4-D tolerance of each line in each environment. In the greenhouse test, seed of each genotype were germinated in petri dishes and transplanted into Super-Cell Cone-tainers® [1] (Ray Leach Cone-tainers, Canby, OR 97013), each holding 150 cm^3 of methyl bromide-fumigated Arredondo fine sand topsoil [loamy, siliceous, hyperthermic, Grossarenic Paleudult (5% silt, 2% clay, 92% sand, 1% organic matter, pH 6.1)]. The experimental design was a 4 replicate, split-plot with rates of 2,4-D as main plots and genotypes as subplots (4 plants/subplot). Four weeks after planting, seedlings were inverted and immersed in 500 mL of the respective 2,4-D solutions for 3 s. The concentrations of 2,4-D were 0, 0.01, 0.05, 0.1, 0.5, 1.0 and 5.0 mM. Each solution consisted of 9.5% (v/v) ethyl alcohol and 0.1% (v/v) X-77 surfactant (alkylarylpolyoxyethlene glycols, free fatty

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[1] Mention of a trade name is only for the purpose of identification and does not constitute a warranty to the exclusion of other suitable substitute products.

acids and isopropanol). At the time of treatment, a control plot for each genotype was harvested so growth could be examined only over the treatment period. Four weeks after treatment, above ground plant parts were harvested, dried at 50 °C for 48 h, then weighed. Dry weights were converted to percent inhibition, using the growth of the control plots during the treatment period as the standard.

The design of the tissue culture experiment was also a 4 replicate split-plot with rates of 2,4-D as main plots and genotypes as subplots (4 hypocotyls/subplot). Seed of the various genotypes were surface sterilized by immersion in concentrated H_2SO_4 (16 M) for 5 min. The seed were then immersed in saturated aqueous $Ca(OCl)_2$ for 5 min, and then rinsed 10 times in sterile deionized water. Seed were then plated into 15 x 100 mm sterile disposable petri dishes containing 25 mL of SGL nutrient medium for a source of aseptically-grown seedlings (Phillips and Collins, 1979). After germination and expansion of cotyledonous leaves, 10 mm long hypocotyl sections were dissected from the seedlings and plated into 20 x 100 mm petri dishes containing 50 mL of L2 nutrient medium solidified with 8 g L^{-1} agar (Phillips and Collins, 1979). Seven 2,4-D treatments (0, 0.001, 0.01, 0.05, 0.1, 0.5, and 1.0 mM) were utilized and were mixed directly into the L2 medium prior to autoclaving. The auxin-type herbicide picloram

(0.06 mg L^{-1}) and the cytokinin 6-benzylaminopurine (0.01 mg L^{-1}) were included as regular components of the nutrient medium. Petri dishes were wrapped with film and stored on shelves at 25°C under low-intensity (50 umol photon m^{-2} s^{-1}) fluorescent plant lights (16 h light/day). Six weeks later, callus growth was dried, weighed, and data were converted to percent inhibition.

Data in both experiments were analyzed initially by analysis of variance to test model significance. Regression analysis was performed on percent inhibitions and log-converted rates. Best-fitting linear or quadratic relationships were chosen according to F-values, R^2 values, and significance of parameter estimates. Regression equations were developed to calculate I_{50} (50% inhibition) values for each genotype in each test. The predictive I_{50} values were then used as a measure of degree of toxicity. I_{50} values were ranked from highest to lowest in each experiment and a rank correlation was performed (Snedecor and Cochran, 1967). Actual I_{50} values were also compared using simple correlation procedures.

Screening Genotypes for Regenerative Potential

Callus, derived from hypocotyl sections obtained in a manner similar to the one described in the previous section, was used to screen for regenerative ability.

Genotypes from 'Kemstar', 'Arlington', 'Noline', 'Altaswede', 'Redman' and local breeding lines were represented. Callus cultures were placed onto LSE medium, containing 0.01 mg L^{-1} 2,4-D and 2.0 mg L^{-1} adenine for induction of somatic embryogenesis (Phillips and Collins, 1983). These cultures were maintained at 25 °C under low-intensity (50 umol photon m^{-2} s^{-1}) fluorescent plant lights (16 h light/day). Routine subcultures occurred every 3-4 weeks. After approximately 3-4 months, continual visual assessments identified the genotype with the greatest appearance of somatic embryos and qualitative otherwise suited for in vitro selection systems, such as vigorous callus growth.

Selection of 2,4-D Tolerant Cell Lines

A genotype selected from Arlington (R578-OPC, supplied by the lab of Dr. G. B. Collins at the University of Kentucky) was used as the callus line in the selection experiments. Callus cultures of R578-OPC were utilized as explant inoculum to establish suspension cultures (Phillips and Collins, 1980). These suspensions were grown in 125 mL glass flasks that were continuously rotated at 120 RPM at 25 °C and subcultured on a weekly basis. For the selection experiments, 5 mL of the suspensions were transferred using a small bore pipet into 100 x 20 mm disposable petri dishes containing 50 mL of L2 medium

supplemented with 40 mg L^{-1} 2,4-D. Preliminary experiments had established this rate to be approximately 95% inhibitory. Dishes were stored as described earlier. After 2-3 months, those cells that were capable of growth on the selection medium were subcultured onto standard L2 medium for 2-3 months (cycle one tolerant lines). After this time, callus cultures were rechallenged on the L2 medium with 40 mg L^{-1} 2,4-D. Again, only those cells or sections of the callus tissue showing vigorous growth were selected and subcultured onto standard L2 medium (cycle two tolerant lines). These were routinely subcultured every 4 weeks until ready for use in the evaluation experiments.

Evaluation of 2,4-D Tolerant Cell Lines

A 2,4-D bioassay procedure was developed using modifications of standard auxin bioassay techniques described by Nitsch and Nitsch (1956) and Yopp et al. (1986). It was intended that this bioassay be used to quantify levels of 2,4-D tolerance in selected and control callus lines.

Hull-less oat (Avena sativa L.) seed (supplied by R. D. Barnett, North Florida Research and Education Center, University of Florida, Quincy, FL 32351) was used in all experiments for the source of coleoptile and internode tissue. Coleoptile tissue was obtained by placing seed on moistened filter paper in petri dishes. The dishes were

placed in the dark and were supplied with 2 h red light/day (G.E. 25W red light bulb emitting 20 umol photon m^{-2} s^{-1}) to suppress the growth of the first internode. The internode tissue was obtained in a similar manner but seed were not subjected to the red light treatment. After 72 h at 22-25 °C, both sets of seedlings were ready for experimentation.

Coleoptile sections, 6 mm in length, were uniformly obtained using a tissue segment cutter (Mitchell and Livingston, 1968). The sections were cut 3 mm below the tip of seedlings that were approximately 25 mm in length. The internodes were obtained in a similar manner but sections were cut starting at 2 mm below the coleoptilar node. The coleoptile sections (containing the primary leaf) were rinsed for 1 h in distilled water containing 1 mg L^{-1} $MgSO_4$. The internode sections were rinsed for 1 h in distilled water.

For all experiments, a basal medium was prepared for physical support of ost tissues and buffering potential. The aqueous solution was supplemented with 20 g L^{-1} sucrose, 0.66 g L^{-1} KH_2PO_4, 1 g L^{-1} citric acid, and 8 g L^{-1} agar (pH=5.0). Twenty-five mL (3-4 mm of depth) of this solution was poured into disposable 100 x 20 mm petri dishes. The coleoptile and internode sections could then be placed standing up (apical end up) in the agar-solidified medium. This left approximately 2 mm of

tissue exposed; the top of the tissue could then support either agar blocks or callus tissue.

A standard response experiment was conducted to determine response of tissue to known concentrations of 2,4-D. Agar blocks (approximately 15 mm^3 in size) were cut from distilled water solidified with 20 g L^{-1} agar. These blocks were carefully placed on the apical end of either the coleoptile or internode sections supported by the agar in the petri dishes. The blocks were injected with 1 ul of the following 2,4-D solutions: 0, 0.0001, 0.001, 0.01, 0.1, 1.0, 10, 100, 500, and 1000 mg L^{-1}), using 0.95% (v/v) ethyl alcohol. The experimental design was a 3 replicate randomized complete block. Coleoptile and internode sections were treated as separate experiments. Lids were placed on the dishes to prevent dessication and the dishes were placed in the dark at 22 ^{o}C. After 24 h, elongation of the sections was measured using caliper-type micrometers. This test was repeated using similar materials and methods and the data from the two tests were combined.

The red clover callus tissue to be utilized in the two bioassay experiments was selected for tolerance using procedures described in the previous section. As mentioned, they were maintained on standard nutrient medium prior to initiation of these bioassays. For the evaluation of tolerance levels, the callus tissue was challenged on L2

nutrient medium supplemented with 40 mg L^{-1} 2,4-D. Callusae of 20 lines were divided into 0.25 g segments and placed on the medium in each of 4 replications. After 6 weeks, these tissues were subcultured onto nutrient medium supplemented with 10 mg L^{-1} 2,4-D. After 4 weeks on this medium, the tissues were used in the bioassays.

In the first bioassay experiment, fresh callus tissue was used. From each of the replications, a segment of callus tissue was randomly chosen that visually was estimated to weigh 15 mg and have a volume of 15 mm^3. Care was taken to assure that the these callus sections were similar in size, shape and general appearance. These callus sections were then placed directly on top of the respective coleoptile and internode sections standing in the agar plates. The experimental design was a 4 replicate randomized complete block, again testing coleoptiles and internodes as separate experiments. The second bioassay test was similar except that sections of callus tissue were initially frozen at -20 °C for 48 h, then thawed prior to usage. This served to disrupt membrane integrity as a means of preventing the differential release of 2,4-D that may occur in fresh tissue. The storage and measurement of oat sections were as previously described.

The data from the standard curve experiment were analyzed initially by analysis of variance to test model significance. Regression was then used to develop

equations that could predict the concentration of 2,4-D that would cause a certain increase in length of the oat sections. The data from the use of callus tissue were analyzed by analysis of variance and means of treatments were compared to control means using Dunnett's test to detect significant differences (Steel and Torrie, 1960).

Regeneration of 2,4-D Tolerant Callus Lines

Callus lines shown to be tolerant to 2,4-D by the bioassay procedure were subjected to regeneration attempts. Callus tissue, maintained on standard L2 nutrient medium, were subcultured into 100 x 20 mm disposable petri dishes containing 50 mL of LSE medium, supplemented with 0.01 mg L^{-1} 2,4-D and 2.0 mg L^{-1} adenine. These dishes were stored on shelves at 25 °C under low-intensity (50 umol photon m^{-2} s^{-1}) fluorescent plant lights (16 h/day). The cultures were subcultured every 3-4 weeks and observed for appearance of somatic embryos.

Results and Discussion

Correlated Response to 2,4-D

The lines chosen for this correlation study (Table 4-1) represented a variable range of 2,4-D tolerance levels. Some lines were known to be susceptible to 2,4-D in vivo (e.g., Nollie, Kenstar) and others had expressed

Table 4-1. In vivo and in vitro responses to 2,4-D of 8 red clover genotypes with differential tolerances to 2,4-D.

Half-Sib Family or Cultivar	In vivo				In vitro			
	I_{50} +	SE	R^2	Rank	I_{50}	SE	R^2	Rank
	-----mM-----		*		-----mM-----			
L13,10,3	0.53	0.02	0.76	1	0.07	0.02	0.64	2
L9,4,6	0.33	0.03	0.76	2	0.045	0.005	0.74	3
SL9,1,1	0.31	0.03	0.69	3	0.077	0.025	0.57	1
SR8,3,4	0.24	0.01	0 73	4	0.032	0.002	0.87	5
Nolina	0.23	0.02	0.89	5	0.03	0.002	0.89	6
Kenstar	0.21	0.03	0.78	6	0.018	0.001	0.89	7
L8,4a,1	0.18	0.01	0.81	7	0.039	0.003	0.97	4
R30,7,7	0.15	0.01	0.69	8	0.015	0.001	0.45	8

+ I_{50} values are predicted values determined by linear or quadratic regression equations and represent rate of 2,4-D at which 50% growth inhibition occurs.

* All models were significant at $P < 0.001$ level.

increased levels of tolerance. For these lines, there was a significant rank correlation ($r_s = 0.79$, $p = 0.05$) between in vivo and in vitro responses to 2,4-D. There was also a significant simple correlation ($r = 0.77$, $p = 0.02$) between actual I_{50} values. For this type of correlated response to be valid, not only must tolerant whole plants be tolerant in vitro, but susceptible whole plants must also be susceptible in vitro. These data substantiate the validity of this correlation.

These experiments indicate that elevated levels of 2,4-D can have direct toxicity on red clover cells. It was necessary to establish that fact so that ample selection pressure could be applied on the cells; the desired result being the ability to select only 1 to 5% of the cells. These data may also suggest that the tolerance seen at the whole plant level is metabolically based. If the tolerance was based on differential uptake or translocation, for instance, then this would more than likely not translate to the cellular level. Observations on whole plants also tend to support this hypothesis as 2,4-D treated plants all showed the same initial 2,4-D injury symptoms. The tolerant plants were able to recover more rapidly and outgrow the damage, while susceptible plants were not able to recover and usually died. This observation also supports the possibility of differential 2,4-D metabolism as the basis of tolerance, which indicates that it may be selected for at the cellular level.

These data provided sufficient initial justification to use in vitro selection techniques to screen red clover cultures for 2,4-D tolerance. If a genetically based system of 2,4-D metabolism can be selected in vitro, such as that demonstrated at the whole plant level, there is reason to believe that the tolerance would be transmitted to regenerated plants.

Screening Genotypes for Regenerative Potential

Red clover is similar to other legumes in that regeneration from callus tissue is often difficult. Obviously, the success of an in vitro selection system is greatly enhanced if a genotype of high regenerative potential is used. Keyes et al. (1980) and Bhojwani et al. (1984) have shown that variation exists among genotypes of red clover for regenerative ability. This is to be expected for a highly heterozygous, cross-pollinated crop such as red clover. It was therefore necessary to screen a large number of genotypes to identify one type with the greatest regenerative potential. A more desirable situation may be to develop a population of red clover plants with increased ability to regenerate by using recurrent selection, such as that accomplished with alfalfa (Bingham et al., 1975).

One genotype was identified that was superior in several regards. This genotype (R578-OPC) was a selection

out of Arlington, a cultivar adapted to the northern United States (Smith et al., 1973). R57S-OPC is vigorous in callus growth and is readily amenable to suspension culture. In addition, it demonstrated a capacity to differentiate that was not seen in approximately 100 other red clover genotypes that were screened. R57S-OPC was therefore chosen as the primary genotype for the selection experiments.

Selection of 2,4-D Tolerant Cell Lines

Cells in suspension cultures were transferred to agar-solidified selection medium using a small bore pipet so that large cellular aggregates would be excluded. The majority of these cells placed on the selection media were small cellular aggregates that could be uniformly exposed to the surface of the agar. After 2 to 3 months, cellular aggregates could be identified that were capable of survival and growth on the 40 mg L^{-1} 2,4-D medium. These aggregates varied in size and appearance. To avoid selection of non-tolerant lines it was necessary to subject these callus lines to a second cycle of selection after a period of time on standard nutrient media.

These procedures resulted in the isolation of 16 cycle two callus lines with tolerance to 2,4-D. Several lines were not carried through the second cycle of selection so that we could compare the effectiveness of one cycle of selection versus two cycles

The selection systems imposed on these cultures appear to have altered characteristics other than the response to 2,4-D. In comparison to the control tissue, selected callus has become less friable, darker in color and the growth rate of the cultures have been reduced; regardless of the presence of 2,4-D in the medium. The control tissue, having been subcultured over a two year period, also displayed a reduced growth rate, although not to the same degree as the selected tissue. These modifications may diminish the regenerative ability of the controls and the selected lines.

Evaluation of 2,4-D Tolerant Cell Lines

The 2,4-D bioassay procedure that was developed was well suited to evaluation of callus tissue. Small amounts of callus tissue were required and time and labor were kept to a minimum. Each test took only 4 days to complete and only required ½ h of labor. The results (Tables 4-2 and 4-3) provided satisfactory determinations of tolerance levels. Control internodes and coleoptiles (those with no callus) did not show appreciable increases in length. The R57S-OPC callus control, which was never placed on any 2,4-D media, caused coleoptile growth which was 0.5 to 0.8 mm longer than the controls. This can be attributed to the picloram in the standard nutrient media which displays auxin-like properties. The high control, C0-01, got

Table 4-2. Results of two bioassay tests, using elongation of oat internodes as a measure of degree of 2,4-D tolerance of callus lines selected for tolerance to 2,4-D.

Callus Line	Internode Expt. One Length	Rank	Internode Expt. Two Length	Rank	Overall Ranking
Control [a]	7.2 mm	1	6.8 mm	1	1
B578-OFC [b]	7.7	2	7.3	2	2
C0-01 [c]	12.3	21	9.3	21	21
C1-05 [d]	11.7	20	8.8	18	20
C1-08	10.9	14	8.8	19	18
C2-01	10.4	11	8.4	14	14
C2-02	8.0	3	7.8	7	4
C2-03	9.3	4	8.6	17	10
C2-04	10.3	9	7.7	6	5
C2-05	11.0	15	8.1	9	13
C2-06	10.4	12	7.7	5	7
C2-07	10.1	8	8.4	15	11
C2-08	10.4	10	8.2	11	9
C2-10	11.2	17	8.2	10	16
C2-11	11.4	18	8.3	12	17
C2-12	10.0	7	8.3	13	8
C2-13	11.7	19	8.6	16	19
C2-14	10.8	13	7.3	3	6
C2-15	9.5	5	7.6	4	3
C2-16	9.7	6	9.0	20	15
C2-19	11.2	16	7.9	8	12
Dunnett's [e]	0.5 *		1.1 *		

* Treatments are significantly different from the high or low controls if they differ by this value, according to Dunnett's test at $p = 0.05$.

[a] A low control, no callus tissue was placed on internode sections.

[b] A low control; standard callus tissue that has never been on media supplemented with 2,4-D.

[c] A high control; callus tissue that has not been through any cycles of selection yet was challenged on 2,4-D media for bioassay evaluation tests.

[d] C1 prefixes indicate these callus tissues have been through one cycle of selection for 2,4-D tolerance.

[e] C2 prefixes indicate these callus tissues have been through two cycles of selection for 2,4-D tolerance.

Table 4-3. Results of two bioassay tests, using elongation of oat coleoptiles as a measure of degree of 2,4-D tolerance of callus lines selected for tolerance to 2,4-D.

Callus Line	Coleoptile Expt. One Length	Rank	Coleoptile Expt. Two Length	Rank	Overall Ranking
Control[a]	7.8 mm	1	7.5 mm	1	1
R576-6[b]C	8.5	2	8.2	2	2
C0-01[c]	10.6	14	10.8	21	21
C1-05[d]	10.5	12	9.9	16	16
C1-06	10.2	8	9.6	15	11
C2-01	10.8	18	9.0	8	15
C2-02	10.3	9	8.8	7	4
C2-03	9.8	5	10.4	19	13
C2-04	10.7	17	8.8	6	10
C2-05	9.7	3	10.1	17	7
C2-06	11.3	20	8.4	3	9
C2-07	11.1	19	9.4	13	20
C2-08	10.7	16	9.1	9	14
C2-10	10.5	13	9.3	11	12
C2-11	11.6	21	9.2	10	19
C2-12	9.9	6	9.3	12	6
C2-13	9.8	4	8.5	14	5
C2-14	10.1	7	8.4	4	3
C2-15	10.6	15	8.4	5	8
C2-16	10.4	10	10.5	20	16
C2-19	10.5	11	10.3	18	17
Dunnett's	0.7*		0.6*		

* Treatments are significantly different from the high or low controls if they differ by this value, according to Dunnett's test at p = 0.05.

[a] A low control; no callus tissue was placed on coleoptile sections.

[b] A low control; standard callus tissue that has never been on media supplemented with 2,4-D.

[c] A high control; callus tissue that has not been through any cycles of selection yet was challenged on 2,4-D media for bioassay evaluation tests.

[d] C1 prefixes indicate these callus tissues have been through one cycle of selection for 2,4-D tolerance.

[e] C2 prefixes indicate these callus tissues have been through two cycles of selection for 2,4-D tolerance.

selected for tolerance but challenged on 2,4-D media for the evaluation tests, was the tissue that caused the greatest amount of elongation in three of the four experiments. This would indicate that this tissue has the greatest amount of susceptibility to 2,4-D. It is possible that C0-01 could not metabolize the 2,4-D that it took up, and consequently there were large amounts of parent compound present within the tissue. The average length achieved by the internodes (10.8 mm) and coleoptiles (10.7 mm) corresponds to 0.3 ug and 0.37 ug of 2,4-D, respectively, according to the standard response curve shown in Figure 4-1.

The callus tissue that was subjected to only one cycle of selection (C1-05 and C1-08) proved to also be susceptible in the internode evaluations, ranking 20 and 18, respectively and moderately susceptible in the coleoptile tests, ranking 16 and 11, respectively. These results establish the importance of two cycles of selection. The cycle two selections showed variable responses to the 2,4-D. As would be expected, not all lines responded in a similar manner and individual lines varied in response from test to test. Not all lines would be considered tolerant, as some lines in some tests equalled the amount of elongation shown by C0-01. One of the more tolerant lines in the coleoptile test, C2-14, had an average length of 9.2 mm. This corresponds to 0.05 ug

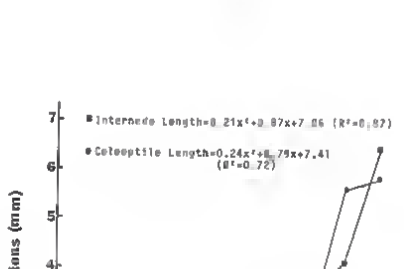

Figure 4-1. Response of oat coleoptile and internode sections to known concentrations of 2,4-D which were injected into agar blocks resting on the apical end of upright oat sections.

of 2,4-D or an 86% decrease in 2,4-D concentration compared to the C0-01 control. In the internode test, C2-15 had an average length of 8.5 mm, corresponding to 0.01 ug of 2,4-D. This would indicate that the line contains 97% less 2,4-D than the C0-01 control.

There was moderate agreement between the results of all tests. The exception is the first coleoptile test, where the results were not comparable with the other three tests. There was not a significant correlation between the two coleoptile tests. This is likely due to experimental procedure. The first coleoptile test was the first experiment completed and consequently was used to define procedures. There may also have been differences related to using different tissue types (fresh vs. frozen) in the two tests. The two internode tests, in contrast, were correlated ($r=0.84$, $p = 0.001$). The rank correlation between the overall placement of the callus lines in the internode and coleoptile tests was also high ($r=0.62$, $p = 0.01$).

These results show that this bioassay procedure can be effectively utilized for evaluation of 2,4-D tolerance levels in callus cultures. The internodes provided more repeatable results. The tests may be more effective in detecting wide differences, such as between susceptibility and tolerance but will require refinement to be able to definitively discriminate between subtle differences in tolerance levels.

Regenerstion of 2,4-D Tolerant Cell Lines

Red clover does not differentiate easily under the most ideal conditions. After subjecting the callus lines to 2 cycles of selection and having subcultured many of them for a time period exceeding 2 yr, they undoubtedly have an even more diminished capacity to regenerate. Also, since low levels of 2,4-D are used as a supplement in the red clover nutrient medium to help induce morphogenesis, it is entirely possible that the 2,4-D tolerant callus tissue will not differentiate under those conditions. Indeed, these lines have not responded to regenerstion attempts Four months on the regeneration medium have not induced any of the tolerant lines to differentiate. Efforts are still underway, but we remain skeptical of the possibilities of regeneration and alternative regeneration procedures are being explored. Without regenerated plants, it is not possible to determine if the 2,4-D tolerance is genetically based.

Comparison Between Conventional and Cellular Selection

In vitro selection has been regarded as a tool that plant breeders can use to reduce the amount of time spent screening plants in the field. This may certainly be true but with some stipulations. If one is working with inbred lines and is interested in finding a single gene trait, then thousands of plants may have to be screened before

isolation of that gene. In vitro selection could be more effective and efficient than whole plant screening in that situation, but only if the crop species in question is a reliable regenerator. More effort needs to be placed on developing regeneration systems for all crop species to insure that this method can be used productively. A second situation that might favor in vitro selection is one where whole plant variability has been limited and new sources of variability are needed. Again, this is more plausible with inbred lines and reliable regenerators.

Cross-pollinated, highly heterozygous crops, such as red clover, provide a different scenario. Even if a particular genotype or population has not been found that possesses certain desirable characteristics, these can often be developed through recurrent selection. Phenotypic recurrent selection is a powerful breeding tool that should enable breeders to develop desirable populations in many instances without resorting to alternate sources of variation such as somaclonal, mutation, interspecific hybridization, etc. (Taylor et al., 1985). The amount of variation that can be developed through recurrent selection is theoretically unlimited. The previous chapter illustrated the successes of a recurrent selection program to increase levels of 2,4-D tolerance. These results exceeded those obtained through tissue culture during the same time frame.

An important consideration before undertaking any breeding method is investigating how the trait may be genetically controlled. The answer may provide insight into which selection method will be more suitable. Recurrent selection can be effective when traits are controlled either qualitatively or quantitatively. In vitro selection has been most effective when the trait was controlled qualitatively, and probably will have little value when selecting for multigene traits. When one has no preconceived notion or prior knowledge of the heritability of the trait, such as our situation with red clover and 2,4-D, then the less risky crop improvement method may prove to be recurrent selection.

A common criticism of conventional breeding is that it is very labor-intensive. Our recurrent selection system did prove to be more laborious than the in vitro selection system, yet one can not argue with the results achieved. On an economic return basis, the conventional approach gave better crop improvement results for the money spent. A plant breeder should be willing to take the necessary steps to accomplish goals, even if it requires more labor. While in vitro selection was less laborious, these advantages were overridden by the failure to regenerate plants.

The agronomic potential of the 2,4-D tolerant red clover needs to be further investigated. Another

difficulty with recurrent selection is selecting for one trait without simultaneously introducing other undesirable characteristics into the population (Taylor et al., 1985). The same, however, can be said for the products of tissue culture. Somaclonal variation may prove to be disadvantageous in certain instances as it may cause unwanted changes in elite lines. If a trait can be successfully selected in vitro, then equal amounts of time must be spent placing that trait in suitable genetic backgrounds and evaluating those lines for agronomic potential (Bingham, 1983).

Self-incompatibility systems and inbreeding depression in crops such as red clover also pose significant obstacles in the practical application of the products of an in vitro selection system. Due to inbreeding depression, a few 2,4-D tolerant regenerated plants would have limited agronomic value. Unless the 2,4-D tolerance could be easily backcrossed into a synthetic population, large numbers of plants with differing genetic backgrounds would be required to develop a usable 2,4-D tolerant population. A very efficient cell to plant regeneration system involving many genotypes is a necessity for this to be plausible.

Grenzal (1979), promoting in vitro selection, has stated that one of the reasons for the few reports of successful in vivo selection for herbicide tolerance is

the problem of finding ways to exert sufficient selection pressure to obtain tolerance. This may be a problem with self-pollinated crops but has not proven to be problematic with our recurrent selection system on a cross-pollinated species. Greenhouse screening provides a fairly uniform environment and thousands of plants can be efficiently evaluated. Recurrent selection is a tool that does not require greater than a 95% kill of plants to be successful. Since selections can be made on a relative basis, exertion of intense selection pressures are not absolutely necessary to provide satisfactory progress, as shown by our research.

In summary, the comparison between conventional and cellular selection for 2,4-D tolerance in red clover favored the conventional method, as better results were obtained. Each breeder must examine their own situation and then decide the most effective method of crop improvement to suit the breeding objectives. Each breeding method has its own advantages and disadvantages, which can be argued indefinitely. The breeder who can use the advantages of each for the greatest benefit will be most productive in crop improvement.

CHAPTER V

RESEARCH SUMMARY AND CONCLUSIONS

This research examined two different methods of selecting for tolerance to the herbicide 2,4-D in red clover. Four cycles of recurrent selection were conducted at the whole plant level and two cycles of in vitro selection were accomplished. Despite being more laborious, the conventional breeding method proved to be more reliable than tissue culture selection. The recurrent half-sib family selection procedure resulted in a 35% increase in level of 2,4-D tolerance. There are no indications that there is a limit to the amount of further progress that can be made. Future research should focus on further increasing the levels of 2,4-D tolerance, with attention also given to the agronomic potential of the populations. This project also lends itself to studies aimed at determining mechanisms of 2,4-D tolerance.

In vitro selection successfully isolated callus lines that had increased levels of 2,4-D tolerance. In vitro selection was accomplished with a relatively minor amount of labor but required more operating expense capital than the recurrent selection procedure. A simple bioassay

procedure was developed that enabled the determination of estimates of the amount of 2,4-D present in callus tissues. It was shown by the bioassay test that two of the more tolerant lines contained 86% and 97% less 2,4-D than the susceptible control tissue. The advantages of tissue culture selection were overridden by the failure to regenerate plants. Future research, in red clover and similar crop species, should be aimed at enhancing the regeneration potential of callus tissue. Also, studies can be conducted in conjunction with whole plant research to examine tolerance mechanisms.

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BIOGRAPHICAL SKETCH

Steven Glenn Taylor, son of Dr. Norman and Evelyn Taylor, was born July 25, 1959, in Lexington, Kentucky. He graduated from Lexington Tates Creek Senior High School in 1977 and entered the University of Kentucky the same year. In 1981, he received the Bachelor of Science degree in entomology. After graduation, Steven owned and operated a private agricultural consulting firm for two years in Henderson, Kentucky.

In June 1983, he entered the Graduate School of the University of Florida, and earned a Master of Science degree in agronomy (plant breeding) in December, 1984. In January of 1985, Steven began work towards a Doctor of Philosophy degree in agronomy (plant breeding), also at the University of Florida. Upon graduation in August, 1987, Steven will be employed by Busch Agricultural Resources, Inc. as a rice breeder in Jonesboro, Arkansas.

Steven was married to the former Belinda McCoy of Philpot, Kentucky, on August 13, 1983. They have one son, Ryan Osborne Taylor, born January 25, 1986.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

K. H. Quesenberry

Kenneth H. Quesenberry, Chairman
Professor of Agronomy

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David D. Baltensperger

David D. Baltensperger
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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